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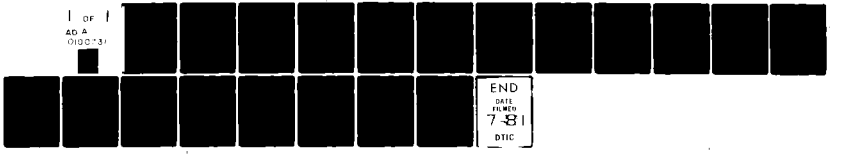

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REPORT NUMBER 2

TARGET ORIENTED DRUGS AGINST *LEISHMANIA*

(Second Annual Summary Report)

URI ZEHAVI, Ph.D.

and

JOSEPH EL-ON, Ph.D.

supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD17-80-G-9474

(previously, Contract No. DAMD 17-79-G-9452)

Faculty of Agriculture
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20 ABSTRACT (Continue on reverse side if necessary and identify by block number)

1. Excreted Factor (EF) is a carbohydrate-rich protein excreted by different strains of *Leishmania*. It has antigenic properties similar to those of the parasite and plays a role in the infective process.

2. Isolation and purification of EF is necessary for:
   (a) study of its biological function

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(b) the use of EF for diagnostic purposes
(c) the use of EF in immunization experiments
(d) the study of the biosynthesis of EF
(e) the preparation of inhibitors of particular biosynthetic steps of EF.

3. Purification of EF by affinity chromatography was markedly improved by introducing *Ricinus* lectin (specific for galactose) column. This enabled us to obtain more reliable amino acid and sugar analysis and will be instrumental in more advanced physical, chemical and immunological studies.

4. We have developed a radioimmunoassay for leishmaniasis utilizing purified EF. The assay can distinguish between *Leishmania* strains and once further developed, should prove most valuable for the diagnosis of the disease.

5. EF plays a role in the infective process of *Leishmania*. We have now shown that surface carbohydrate, related to EF, plays a role in the initial attachment of *Leishmania* promastigots to macrophages - a stage that is a prelude to their engulfment by the macrophages followed by multiplication in their cells.

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   (a) study of its biological function
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TABLE OF CONTENTS

1. Introduction

Excreted Factor (EF) is a carbohydrate-rich protein excreted by different strains of *Leishmania*. Our first annual report has demonstrated the presence of very high galactose content in EF, a property that could be shared also by the surface carbohydrates of the parasite. EF has immunological properties similar to those of the intact parasite and may play a role in the infective process. After it was shown to be species-specific, EF became the basis for serotyping *Leishmania* strains, and is a valuable tool in diagnostic, demographic and ecological studies of the disease (2). The diagnostic methodology, however, is generally insufficient and the development of a reliable radioimmunoassay (RIA) seems most valuable.

2. Purification of EF

EF is produced in relatively small quantities by *Leishmania* promastigotes in culture. Purified EF was required for (a) RIA development, (b) attachment studies and (c) chemical and physical analysis. More advanced chemical and physical studies were therefore hampered by difficulties to scale up the isolation of EF from culture media. This obstacle is now more successfully overcome by directing additional technical help to this issue.

(i) Isolation and phenol extraction - *L. tropica* and *L. donovani* promastigotes were cultivated in Lit medium supplemented with 10% foetal calf serum and antibiotic 100 µg streptomycin and 100 µ penicillin per ml. Cultures at the logarithmic phase of the growth were centrifuged 7 min at 1500 xg. The supernatant was removed and concentrated to 1/10 the original volume and dialyzed for 3 days against distilled water. The dialyzate was concentrated again and extracted with phenol. The aqueous phase was dialyzed for 3 days against distilled water and the dialyzed concentrated EF was fractionated on Sephadex G-100 column. The fractions containing EF as detected by immunodiffusion were pooled, concentrated by freeze-drying and dialyzed again for 3 days against saline. This procedure representsa refinement of that described in the first annual report, §4a.

(ii) Affinity chromatography (*Ricinus* lectin column)

In a typical experiment, 16 mg of EF (§2i) were applied to a *Ricinus* lectin column (Agarose-*Ricinus communis* Agglutinin 120, 1.1 mg protein per ml resin, 7 cm. long, 1 cm. in diameter). The column was first eluted with 0.02 M sodium phosphate buffer, pH 7.0 (12 fractions) followed by a similar buffer containing 50 µ M galactose (all fractions contained 2.6 ml per fraction). The fractions containing EF were detected by immunodiffusion (fraction 14-16), pooled, dialized against water and lyophylised to yield purified EF (2.0 mg) possessing 8 x increase in the specific antigenic activity.

This technique appears to be fast and more efficient than previous techniques for the purification of EF.

3. Chemical Analysis of EF Preparations

Amino acid analysis and monosaccharide analysis were carried out on

samples of EF, purified by affinity chromatography (§2ii) following acid hydrolysis or methanolysis and trimethylsililation, respectively.

(a) Amino acid analysis - EF L137 was analyzed following acid hydrolysis using amino acid analyzer (LKB Model 3201). See Table 1 for results.

Table 1

Amino acid analysis of EF L137

Amino acid	Molar %[a]	Relative number of amino acid residues
Lysine	5.8	1.65
Histidine	3.16	0.9
Arginine	2.83	0.8
Aspartic acid	7.29	2.07
Threonine	22.99	6.55
Serine	16.59	4.73
Glycine	9.57	2.73
Alanine	10.35	2.95
Half cystine	13.54	3.86
Isoleucine	3.51	1.0
Leucine	4.13	1.18
Glucosamine		4.76
Galactosamine		1.81

[a] Total not including glutamic acid, proline and hydroxyproline amounting to 13-44% (not separable in this run).

In the first annual report, §5a, we have commented on the particular features of the amino acid analysis. Here we find some deviations obviously related to the (ca. 6 x) purification that we have achieved. Characteristically, aromatic amino acids are entirely absent.

(b) Carbohydrate analysis - Monosaccharide components of EF were determined by GLC following methanolysis and trimethylsililation (3).

Table 2

Monosaccharide composition of various EFs

Sugar[b]	EF L137 I rel. to mannose[a]	EF L137 II rel. to mannose[a]	EF L137 III rel. to mannose[a]	EF L137 III nmol/mg	EF L137 III %	EF L52 rel. to mannose[a]	EF L52 nmol/mg	EF L52 %
Arabinose	0.1		0.05	8	0.1			< 0.1
Fucose	1.1	1.6	1.1	170	2.8	0.61	188	3.1
Xylose[c]	1	1.3	0.2	29	0.4	0.1	32	0.5
Mannose	1	1	1	154	2.8	1	293	5.3
Galactose	3.2	4.2	4.1	628	11.3	2.2	643	11.6
Glucose[c]	2.8	1.8	0.8	124	2.2	0.6	169	3
Total sugar					33			37.3
Total sugar by Dubois					28			26

[a] Amount relative to mannose (Man = 1.0).

[b] Glucosamine and galactosamine are presented in Table 1.

[c] A large proportion of the xylose and some of the glucose may represent a contaminant eluted from the affinity column.

Fucose, a monosaccharide frequently present at the non-reducing end of glycoproteins and glycopeptides was determined here for the first time.

The proportion of arabinose has diminished as the result of the present purification.

Galactose remains the most abundant neutral sugar.

Microheterogeniety, demonstrated, for instance, by the increase of galactose/mannose ratio if EF peaks between early and late fractions can be understood in view of the galactose binding properties of the *Ricinus* lectin column (the sequence of fractions being I, II, III).

4. Radioimmunoassay (RIA) for the Diagnosis of Leishmaniasis

(a) Labeling by ^{3}H-Acetic Anhydride

EF L137 partially purified by phenol extractions and followed by Sephadex

column chromatography (§2i) was acetylated under Schotten-baumann conditions (aqueous sodium hydrogen carbonate) to yield high specific activity labeled EF. Although most of the label precipitated with homologous rabbit antiserum, Sephadex G-100 column chromatography (§2ii) separated the label from the immunological activity. Thus, the entity labeled must have been a peptide component strongly bound to, but separable from, EF.

(b) Labeling by galactose oxidase - sodium borotritiate

EF [13] (§2i) was labeled at the non-reducing terminal galactosyl residues by the galactose oxidase-sodium borotritiate technique (4). A crude sample of labeled EF (1,700,000 CPM) was added to a carrier EF (§2i), 12 mg) and was further purified by affinity chromatography (§2ii). The purified EF contained 6,500 CPM.

(c) RIA employing ^{125}I - protein A

This technique, originally developed in parallel to the labeling procedures described in §4a and §4b has the advantage of using harder radiation (^{125}I vs. ^{3}H) and developed faster into a useful RIA.

The technique, based on the binding of antibody to solid phase coating [illegible], followed by protein A labeling, was used as previously by Avraham [illegible]. The test was done in both polysterene tubes (100mm x 13mm, Falcon, Cockeysville) and 96 wells microplates (Sterilin, England). Coating with EF was done in tubes and microplates either treated with glutaraldehyde or left untreated. EF and glutaraldehyde were diluted in phosphate buffer saline (bbs) pH 7.4 and dilutions of sera and protein A were made in bbs containing 10% foetal calf serum. The assay for anti-*T. cruzi* and *L. donovani* EF was as follows: 100 µl of EF at different concentrations were added to the 96 wells of microplates, either treated or not treated with 0.1% glutaraldehyde. After 24 hr at 4°C different dilutions of antiserum were added. After another 24 hr at 4°C, the unbound antibodies were removed and 100 µl ^{125}I-protein A containing about 50,000 counts/min were added. The reaction was terminated by the addition of 20 µl of 0.1M NaOH. The solution from each well was then transferred to plastic tubes and the radioactivity measured.

For inhibition studies, 0.2 ml of 1:10 dilutions of antisera were mixed with serial dilations of EF at a final volume of 0.2ml. After incubation for 30 min at 37°C and overnight at 4°C, the adsorbed sera were added to the EF coated microplates and the radiactivity was measured.

Results obtained by this method are as follows:

(i) Coating of plate

As mentioned, EF that had been purified by extraction with phenol followed by gel filtration on Sephadex G-100 (§2i) was found to be suitable for binding to tubes and microplates wells. Other preparations obtained by either precipitation and chromatography or by the dissociation of immune complexes, did not bind to the plates and showed the same activity with antibody as untreated

ntrol EF. It appears that the absolute amount of EF coupled t the plate depends essentially on the level of purification and the nature of the medium component which acts as a carrier rather than the amount of antigenic determinants of the EF.

The concentration of EF used for coating the wells was 0.6 to 1.2 ug/ml. Immunodiffusion of this EF with homologous antiserum produced an immuno precipitate at a maximum dilution 1:2. Concentrations exceeding 1.2 mg/ml increased the strength of the binding without affecting the specificity (Fig.1). At concentrations less than 0.35 mg/ml, no antibodies were detected. Almost no differences in extinction values were seen with wells coated or uncoated with glutaraldehyde.

Specificity of the assay

The interaction of antisera with different EFs and the specificity of the reaction is given in Fig. 2. The results obtained indicated that anti-*L. tropica* and anti-*L. donovani* reacted only with homologous EF. Owing to the higher concentration of rabbit anti-*L.donovani* IgG, as measured by immuno-diffusion, higher binding was observed with this antiserum, as compared with anti-*L. tropica* serum at corresponding dilutions.

In most cases, the activity obtained with the heterologous antiserum was almost the same as that of control normal rabbit serum. With antisera diluated at 1:10, 18% of the anti-*L. donovani* were bound to *L. tropica* EF, 32% of anti-*L. tropica* antibodies were bound to *L. donovani* EF and 12.5% to 16% of normal rabbit antibodies were bound non-specifically to *L. donovani* and *L. tropica* EF respectively.

Competition study

As indicated in Fig. 3, the reaction of both anti-*L. donovani* and anti-*L. tropica* sera could be completely inhibited by preincubation with homologous EF. 300 µg of *L. donovani* and *L. tropica* EF were sufficient to block all the anti-EF antibodies present in 1 ml of homologous antiserum.

Detection of anti-LF antibodies in mice and humans suffering from cutaneous leishmaniasis

Anti-EF activity in sera from humans infected with *L. tropica major* is shown in Table 3. Of 7 sera examined from patients with active cutaneous leishmaniasis, 5 (62%) showed maximum activity of 1.4 to 2.25 times higher than normal uninfected control.

Serum collected from balb/c mice 40 days after infection with *L. tropica*, was also assayed for anti-LF antibodies. These mice showed well-developed lesions and parasites were detected in both, their lesion and in their spleens. The anti-LF antibody activity detected in these sera was as high as 25.6 times that of a normal non-infected mouse serum.

Table 3. Anti-L.tropica EP antibody activity in sera from patients with cutaneous leishmaniasis. The numbers in brackets are the results compared with the activity measured in normal human serum.

serum dilution	% Binding							
	serum A	serum B	serum C	serum D	serum E	serum F	serum G	control serum
1:5	42,3(12.19)	17,2(0,89)	13.3(0.68)	13.6(0.7)	22.9(1.18)	21.4(1.10)	22.3(1.15)	19.3
1:10	22.8(2.25)	15.6(1.48)	9.3(0.92)	9.9(0.98)	19 (1.9)	14.2(1.40)	16.5(1.63)	10.1
1:25	13.0(2.13)	9.0(1.47)	6.2(1.0)	5.5(0.9)	11.2(1.83)	6.6(1.08)	10.7(1.75)	6.1
1:50	8.3(1.5)	7.9(1.19)	4.4(0.66)	3.9(0.59)	6.7(1.01)	4.0(0.6)	5.3(0.8)	6.6
1:100	5.7(1.03)	4.7(0.85)	3.4(0.61)	2.8(0.56)	4.6(0.83)	2.6(0.47)	3.37(0.6)	5.5

The immunological specificity of leishmanial excreted factors, and their separation from other leishmanial antigens and contaminating medium components were used to provide a more sensitive means of detecting and measuring the antibody response to leishmanial infection. The results obtained indicated that the use of leishmanial EF for RIA will prove valuable in the diagnosis of leishmaniasis. The test is specific and sensitive. Undoubtedly, experience will lead to modification and refinement of the technique according to its specific application. However, sufficient details have been presented to enable its use with regard to the detection of cutaneous leishmaniasis and quantitative measurement of EF levels.

5. Surface Carbohydrate (Related to EF) and the Binding of Leishmania Promastigotes to Macrophages

It is known that liver and peritoneal macrophages have specific galactose-binding receptors (hepatic binding protein, HBP) which are able to bind neuraminidase-treated cells where galactose is the non-reducing sugar as a prelude to their catabolism (6). It was demonstrated recently, however, that the galactose or the *N*-acetyl-galactosamine specificity of HBP is not high and additional monosaccharides (e.g. glucose) can compete, though less favourably, for HBP binding (6a).

The first annual report, §5b, has described the presence of a high galactose content in EF, a finding that was further supported by our current analyses, §5b. In addition, we have demonstrated that promastigotes of both *L. tropica* and *L. donovani* are agglutinated by low concentrations of peanut lectin and *Ricinus* lectin and that dissociation is readily achieved by the addition of galactose. This indicates the presence of oligosaccharides possessing terminal non-reducing galactose as a partial structure of the promastigote surface (first annual report §2a and current results concerning *Ricinus* lectin).

We propose that the host cell (macrophage) membrane might contain a galactose-binding receptor, possibly the well-documented hepatic binding protein (HBP), capable of binding either the parasite or EF. *Leishmania* may thus be attached initially to the macrophage via such a galactose-binding site and be subsequently engulfed by the macrophage.

For attachment study of promastigotes to macrophages, the culture system of Handman & Spira (7) was adapted. Peritoneal exudate cells (PEC), mostly macrophages, from C3H mice were harvested 5 days after I.P. stimulation with 2 ml thioglycolate. On the day of harvest, the cells were collected in McCoy's medium containing 5 units of heparin, 100 µg streptomycin and 100 units penicillin per ml. The cell concentration was brought to 5 x 10^5 cells/ml and 1 ml was plated into each well of the 24 wells microplate. Before adding the PEC, 12 mm diameter sterile coverslips were placed in each well. 24 hours after incubation at 37°C in an atmosphere of 5% CO_2 in air, the medium was changed, removed, and 200 µl of the sugar in the desired concentration made in Hank's balanced salt solution containing 1% albumin (HbSSA) was added. After 15 min at 37°C, the sugar was replaced with 400 ml

of the same sugar, at the same concentration containing 2 x 10^7 washed promastigotes. After a further 10 min at 37°C, the PEC were washed 5 times with phosphate buffer, fixed and stained with Giemsa. Following counting, the parasite attachment index (PAI), (§10) was calculated.

Figure 4 shows that different sugars (at 0.3 - 0.5 M) inhibit the attachment of promastigotes to macrophages. Lactose, Gal-β(1→ 4)Glc, being the most efficient. Lower concentrations of sugars promote attachment while 1 M and higher concentrations are toxic to host (macrophage) cells. Apart from standard error possible (Ca. 10%), the effect of lower saccharide concentrations may be due to additional sugar nutrient. Sugars at concentrations of 0.5 M are non-toxic to both promastigotes and macrophages. Following the addition of 0.5 M saccharide and incubation as described, the cells are washed and give normal infective rates.

6. Conclusions

In our first report (§7) we have proposed that surface carbohydrate may play a role in the binding of *Leishmania* promastigotes to macrophages. Our current experiments (§5) support this hypothesis. Thus, *Leishmania*, an intracellular obligatory parasite, appears to use a cellular mechanism designed *inter alea* to remove damaged cells from blood circulation (6) as a vehicle to enter host cells. Once inside, the LF produced has a protective function, being inhibitory to the host's lysosomal enzymes (8).

Our analytical results (§3) already suggest galacto derivatives as possible biosynthetic inhibitors of EF. As soon as more advanced structural results are available and larger structural elements become known, and in view of our original research proposal, one will be in a better position as far as the design of such inhibitors is concerned.

A most important outcome of our work is a specific radioimmunoassay for *Leishmania* (§4c). Effort is to be dedicated to the standardization of the method - to make it diagnostically useful. Additionally, the scope of the method should be widened to more acute types of *Leishmania*.

7. Proposals for Further Research (detailed proposal submitted along with this report)

(i) Purification and structural work on EF. Here we will be employing the affinity chromatography purification (§2ii).

(ii) Radioimmunoassay (RIA) for *Leishmania*. Standardization of the method. Application to human and animal cases.

(iii) Biological role of EF. Use of labeled LF (§4a and 4b) to localize EF. Role of EF in "conditioning".

(iv) Immunogenicity of EF. Use of MDP, mycolic acid etc. to make EF immunogenic.

(v) Inhibitors. Based on strucutral features of EF, inhibitors to its biosynthesis will be designed.

Fig. 1 The effect of different concentrations of coating L.tropica EF on the binding of antibodies from homologous antiserum at dilution 1:25. The EF showed a precipitating line with whole antiserum at 1000 µg/ml.

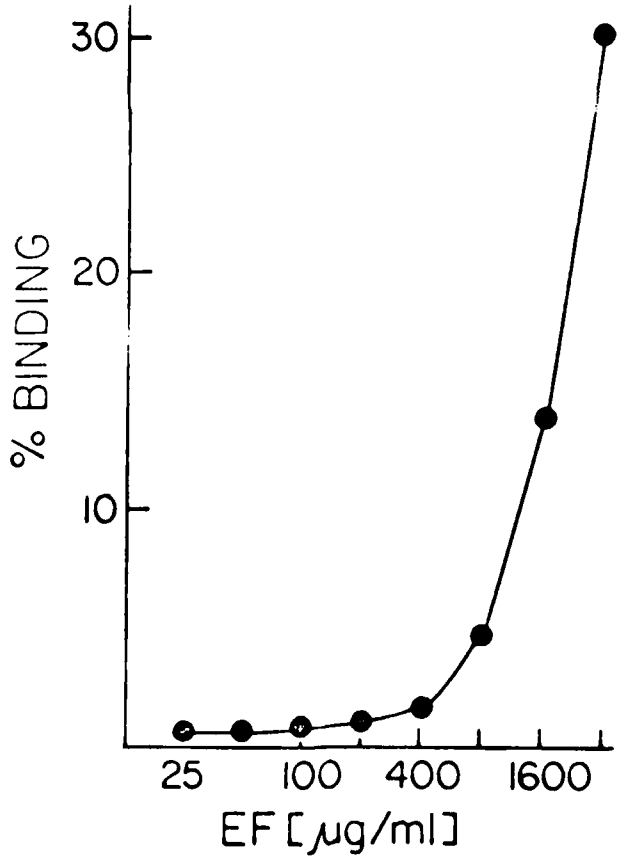


Fig. 2 Binding of ▨ rabbit anti L.tropica antiserum,
□ rabbit anti L.donovani antiserum and
■ normal rabbit serum to plates coated with
L.donovani or L.tropica EF.

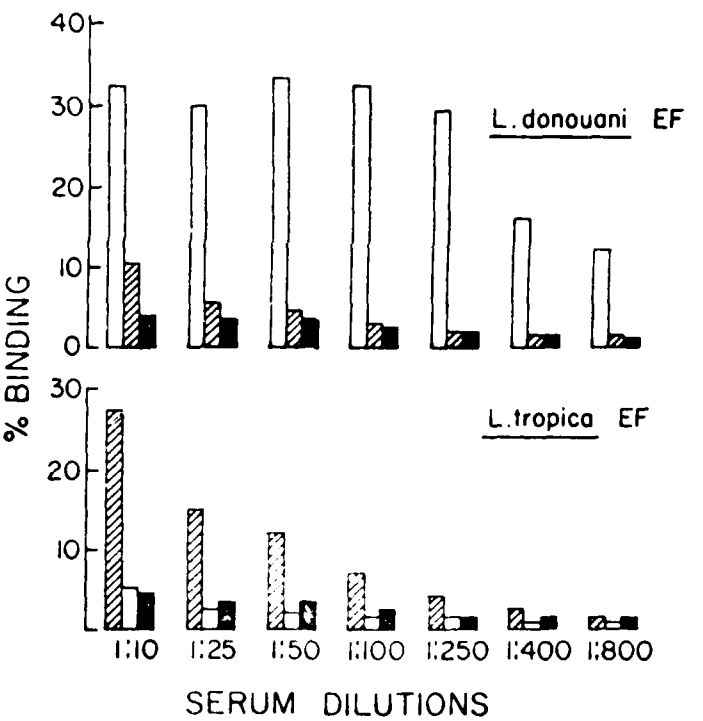


Fig. 3 Effect of absorption of anti L.tropica and anti L.donovani antiserum with homologous and heterologous EF. ● -Anti L.tropica absorbed to homologous EF; ▲ -Anti L.donovani absorbed to homologous EF. The inhibition is calculated by dividing the antiserum absorbed to homologous EF by the same antiserum absorbed to heterologous EF.

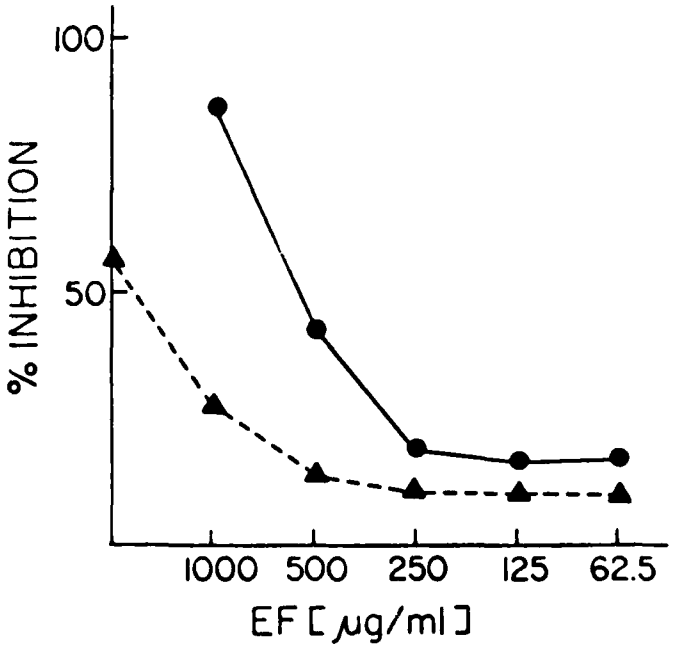
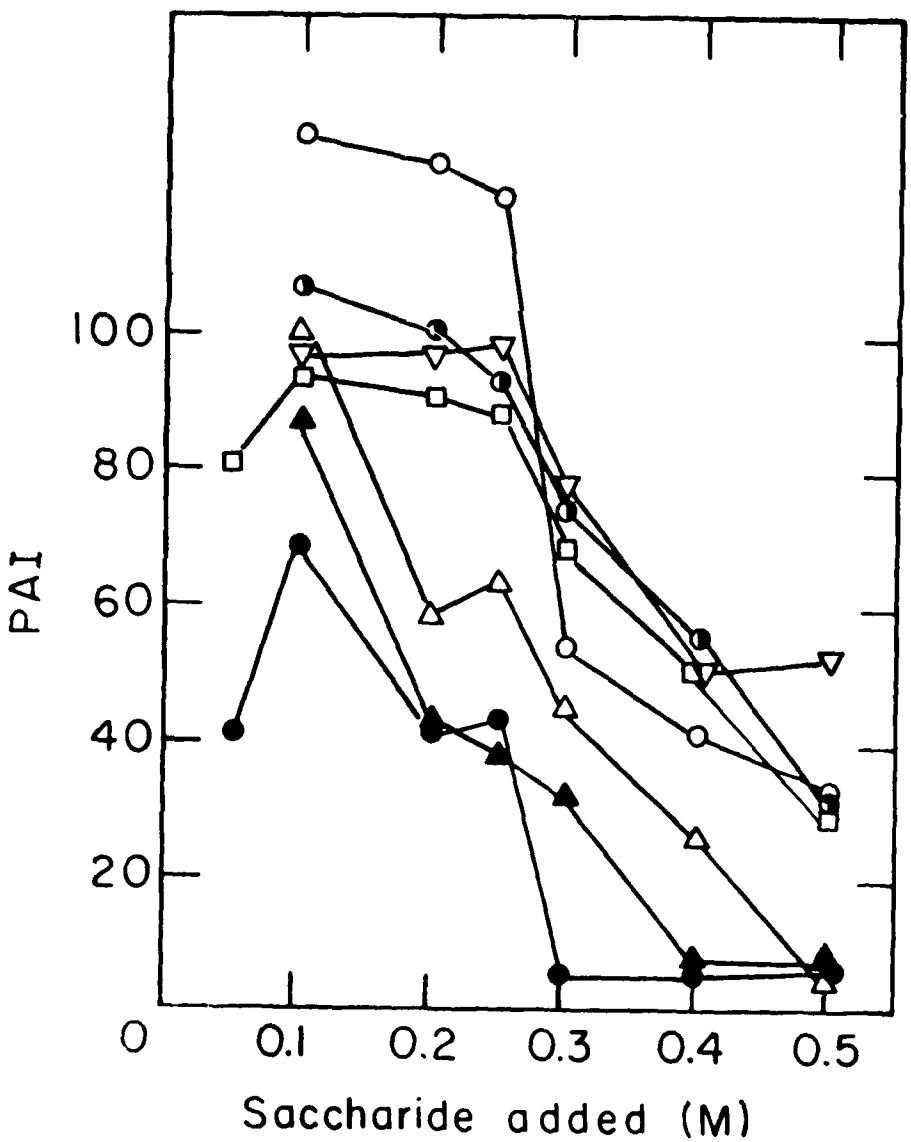


Fig. 4 The effect of sugars at different concentrations on the parasite attachment index (PAI) of *L. tropica* (L 137) to C_3H mouse macrophages.

● - Lactose; ○ - Glucose; ◑ - Methyl α-D-galactopyranoside; □ - Methyl β-D-galactopyranoside; ▲ - Raffinose; △ - Methyl-α-D-mannopyranoside; ▽ -D-arabinose



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10. Glossary

EF — Factor excreted by *Leishmania* (Excreted Factor). EF preparations are designated with digits indicating the leishmanial source.

Leishmanial strains

L137	*Leishmania tropica*	LRC L137
L52	*Leishmania donovani*	LRC L52

These strains were obtained from the WHO *Leishmania* Reference Centre collection maintained in the Department of Protozoology in Jerusalem.

PNA — peanut lectin

RIA — radioimmunoassay

PAI — parasite attachement index =

$$\frac{a_{exp} \times {}^{b}exp \times 100}{a_{cont} \times {}^{b}cont}$$

a = percent of host cells where attachment is apparent. 400 host cells were counted.

b = average no. of attached promastigotes per cell. 100 cells with attached promastigotes were counted.

(i) Figures related to attachment represent a proportion of already engulphed promastigotes.

(ii) Saccharides were included in experiments and avoided in controls.

11. Distribution List

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